Perspective

Differentiation Requires Continuous Regulation

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DIFFERENTIATION has long been conceived of as a series of binary decisions. Waddington's epigenetic landscape (1940) provides a particularly vivid visual image of this concept: differentiation is likened to the path taken by a ball as it rolls down a sloped surface grooved by valleys. Similarly, according to Stuart Kauffman's binary model (1973), early choices limit later probabilities for changes in cell fate that accompany determination and transdetermination. These theories have led to the still widely held view that progression along a developmental pathway entails changes that exclude future possibilities. This view is also embodied in commonly used terms such as "committed stem cell" and "terminally differentiated cell." It suggests that cell states are locked in place by mechanisms that are not easily disrupted.

Two molecular mechanisms that could silence genes that are inappropriate to a given differentiated cell type are "passive" and "active." It seems simplest to silence genes by a "passive control" mechanism: closing down unneeded genes so that they do not require active consideration in a given cell lineage for the life of the organism. Thus, commitment, like Lyonization of the X chromosome, would result in the permanent inactivation of many unnecessary genes. Indeed, what would be the advantage of keeping muscle genes accessible in the liver? Alternatively, differentiation could be governed by an "active control" mechanism: the expression state of each gene being determined by the dynamic interaction of regulatory proteins present in the cell at any given time. This second possibility is often dismissed for the following reasons: (*a*) differentiation appears to be stable, (*b*) plasticity seems unnecessary, and (*c*) the number of regulators required appears cumbersome. In particular, the investment in negative regulators necessary to maintain the majority of genes in a silent state seems disproportionately large. In spite of the appeal of passive control, accumulating evidence suggests that differentiation is stably maintained by continuous regulation, both by positive (Britten and Davidson, 1969), and by negative regulators. We first present the evidence and then the implications of the continuous mode of regulation.

Gurdon's (1962) nuclear transplantation experiments showed that genes were neither lost nor permanently inactivated during development. Upon transfer of an intestinal cell nucleus into an enucleated egg, entire swimming tadpoles developed. However, the frequency of this event was low, unless nuclei were first injected into oocytes (DiBerardino et al., 1986), a step that might allow reprogramming by stripping the DNA of mitotically heritable regulatory influences. Thus, although these experiments provided strong evidence that differentiation was reversible, they did not determine whether genes were silenced by active or passive mechanisms in the course of development. Cell fusion experiments showed that gene activation did not require ooplasm. Genes typical of other differentiated cell types, such as muscle genes in a liver cell, or adult globin genes in a fetal erythroid cell, were transcribed in heterokaryons (Blau et al., 1985; Baron and Maniatis, 1986). Moreover, the activation of silent genes occurred without DNA replication or a replay of the hierarchy of regulators characteristic of development from a fertilized egg to a differentiated tissue. Recently, the constitutive expression of a single cloned regulator, MyoD, was shown to activate the silent muscle genes, myosin heavy chain and desmin, in a range of nonmuscle cell types (Davis et al., 1987). Indeed, in fibroblasts, MyoD caused a complete phenotypic conversion: a muscle-specific distribution of organelles and pattern of gene expression was stably inherited. A muscle phenotype was also induced in more distantly related cell types when MyoD was presumably combined with additional regulators (Schaefer et al., 1990). These experimental manipulations demonstrate that silent genes in "committed" differentiated cells, are readily accessible suggesting that their repression is not passively, but actively controlled.

Critical to the active control hypothesis is evidence that the continuous activity of positive and negative regulators is required to maintain differentiation in the course of normal development. A clear test would show that a disruption in expression of a nodal, or key, regulatory gene alters the fate of cells that are already differentiated. Two types of elegant experimental system have made this test possible: temperature-sensitive mutants and somatic mosaics. Both examine the temporal window during which a particular gene product is required by interfering with its expression, either through a shift in temperature or by x-ray-induced mitotic recombination or chromosome loss. In *Drosophila* and *Caenorhabditis elegans* such experiments have shown that unless regulatory gene expression is continuous throughout adult development, sexual characteristics, such as production of sperm and synthesis of egg yolk proteins, are lost (Kimble et al., 1984; Belote et al., 1985). Similarly, neural cell identity and pattern formation are altered even at late larval stages by disruption of the expression of critical genes, including members of the large polycomb family that encode negative regulators (Duncan and Lewis, 1982; Way and Chalfie, 1989). Perhaps the most striking example of plasticity is found in the adult *Drosophila* central nervous system: a female will engage in a complex male courtship behavior if exposed to a shift in temperature that disrupts the expression of the *tra-2* gene (Belote

and Baker, 1987). These and many other experiments show that the uninterrupted expression of negative and positive regulators is essential to the expression of the differentiated state in vivo.

If differentiation is actively regulated, the stoichiometry, or relative concentration of positive and negative regulators, must play a critical role in its expression at any given time. The effective concentration of a regulator is altered not only by changing its rate of synthesis or degradation, but also by altering the concentration of the proteins with which it interacts. Recent evidence indicating that many regulatory proteins form complexes, for example, heterodimers via leucine zipper or helix-loop-helix motifs (Landschulz et al., 1989; Murre et al., 1989) provides a molecular basis for active regulation of differentiation. Such interactions either promote or inhibit the function of a regulator: the E12 protein enhances and the Id protein prevents the transcription factor MyoD from binding to DNA with maximum efficiency (Benezra et al., 1990). Clearly, in addition to abundance, the relative affinity and cooperative interactions of regulators not only as heterodimers, but also as multimeric complexes (Lin et al., 1990) will have a profound impact on gene expression. Because regulators act in combinations, small changes in the relative concentration of a single component can have large effects on the expression of the cell's differentiated state, by shifting a critical balance, reaching a threshold, and setting off a cascade of subsequent events. Thus, the dosage of genes encoding the helix-loop-helix proteins *daughterless*, *hairy*, and *achaete-scute* determines sex in *Drosophila* (Parkhurst et al., 1990). Gene dosage is also responsible for several human genetic diseases (Epstein, 1986), determining neurosensory cells in *Drosophila* (Botas et al., 1982), and gene expression in cell hybrids (Blau et al., 1985).

As mentioned at the outset, continuous active control poses problems for differentiation. How can the requisite number of regulators be produced? How are stability and memory ensured? These problems are interrelated. A key question is whether the expression of the entire hierarchy of regulators that led to the establishment of a differentiated state is also required to maintain it. That this is not essential was suggested by heterokaryon experiments and made clear by the recent discovery that in eukaryotes, as in prokaryotes, nodal regulators including *Drosophila* homeotic selector gene products (Kuziora and McGinnis, 1988), the signal transducer *c-jun* (Angel et al., 1988), and the helix-loop-helix family of myogenic regulators (Thayer et al., 1989) can activate their own transcription. Thus, sequential gene expression can eventually lead to autoregulation of nodal regulators that now maintain their own critical threshold concentration. By circumventing the regulatory hierarchy, autoregulation limits the number of regulators required to maintain the differentiated state. In addition, autoregulation provides stability and memory. Apparent redundance, or the discovery that many nodal regulators coexist as families of proteins with overlapping functions that can activate each others' expression (for review, see Olson, 1990), ensures that levels of regulators will be maintained and the phenotype of a cell stably remembered without recourse to a passive control mechanism.

When are passive control mechanisms used? The silencing of genes that results from X chromosome inactivation or as a consequence of imprinting is passively controlled. These mechanisms play a critical role in early development, apparently ensuring the balanced contribution of male and female genomes (Thomson and Solter, 1988), rather than silencing the expression of genes as tissues and organs develop. For X chromosomes, it is clear that only one of a pair of genes is active in the same nucleus, an expression state that is established early in embryogenesis and stably transmitted to all progeny cells as methylated heterochromatin. This expression state is not subject to regulation by a change in the balance of *trans*-acting factors during development; it is altered only in the germline. Thus, these regulatory decisions are relatively permanent, persisting for the life of the organism. In contrast to the fixed repression of genes that accompanies X chromosome inactivation or imprinting, the repression of genes typical of differentiation appears plastic and dynamic. This is true even when gene inactivity is associated with changes in "chromatin." Thus, in contrast to previous models (Brown, 1984; Weintraub, 1985), it is now clear that in the absence of DNA replication, inactive genes become hypomethylated, nucleosomes are displaced, and DNAse hypersensitive sites are induced (Sullivan and Grainger, 1987; Bresnick et al., 1990). These changes, which alter the expression state of tissue-specific genes, are readily reversible and can all be accounted for by a change in the stoichiometry of *trans*-acting factors (Grunstein, 1990). Thus, although often collectively referred to as chromatin, the passive forms of gene silencing established early in development are likely to differ at a molecular level from the active forms involved later in the course of differentiation.

Why should the differentiated state be controlled by mechanisms that are dynamic and reversible? Perhaps active control is an evolutionary vestige: a single jellyfish cell can generate numerous different cell types and axolotls can regenerate entire limbs. On the other hand, active control may provide essential plasticity. That plasticity is necessary is clear from recent findings that the same regulatory genes are used at different times in development to specify quite different processes. In addition, recent evidence suggests that differentiation may not be as rigidly determined as it appears. Upon injury to tissues, cells can undergo marked changes in state, or transdifferentiation. Moreover, in the course of normal development, cells like those of the neural crest, give rise to a multiplicity of unexpected cell types, including representatives of different embryonic germ layers (Le Douarin, 1986). If the fate of individual cells is followed using novel cell lineage markers, additional plasticity may be uncovered.

What has become of Waddington's epigenetic landscape? The differentiated cell, instead of being caught in a groove, appears to require continuous control to prevent it from wandering into another valley. Although gene rearrangements and loss are the norm in malignant cells, it is striking how few changes are completely irreversible in differentiated cells, the DNA changes that lead to immunoglobulin expression being a marked exception. Indeed, in theory, any nucleus exposed to the appropriate constellation of proteins should be able to perform functions typical of any given differentiated cell type. For further progress, it will be important to distinguish actively from passively regulated chromatin at a molecular level. What is the nature of the *trans*-acting factors that displace nucleosomes and cause chromosomes to loop? How are the genes on the X chromosome permanently inactivated? Although autoregulation of regulators plays a

key role in stability, there are likely to be additional mechanisms for establishing memory. What are these mechanisms and how are they actively maintained and yet reversed with relative ease? Indeed, with additional research, it may eventually be possible to select a specific valley and channel Waddington's ball at will.

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